Chapter 7

General Discussion
The intestine, consisting of both the small intestine and large intestine or colon, is a unique tissue which needs to maintain a delicate balance between immunological tolerance to harmless antigens and launching immune responses to harmful pathogens. The mucosal immune system contains a variety of organized gut associated lymphoid tissues (GALT) composed of B cell follicles, T cells and dendritic cells (DCs). These structures provide a unique environment for bringing these specialised immune cells into close contact. This gives the intestines local structures in which immune responses can occur. In the healthy setting the small intestine contains Peyers Patches (PPs), solitary intestinal lymphoid tissue (SILT) and the draining mesenteric lymph node (MLN). PPs and MLNs are secondary lymphoid tissue and have been shown to be pre-programmed to form at predestined sites during embryonic life. SILT on the other hand is referred to as inducible lymphoid tissue due to the fact that they form after birth and are detectable in the small intestine around two weeks after birth. Both secondary lymphoid tissue and SILT are thought to be dependent on the lymphotoxin (LTαβ) - lymphotoxin beta receptor (LTβR) signaling pathway as numerous research groups have reported the absence of these structures in the context of the adult intestine of LTα-/- and LTβR-/- 3-5. As most research focuses on the formation of secondary lymphoid tissue and SILT in the context of the small intestine less is known about secondary lymphoid tissue and SILT in the context of the colon. In the chronic inflammatory setting of the colon a third type of lymphoid tissue may emerge i.e. tertiary lymphoid tissue. Due to the fact that secondary lymphoid tissue and SILT already exist in the healthy colon it is a challenging task to identify whether in addition tertiary lymphoid tissue forms in response to inflammation. Tertiary lymphoid tissue occurs in a variety of chronic inflammatory diseases and at present it is largely unknown if these tertiary lymphoid tissues are of benefit or detriment to the host.

**Secondary lymphoid tissue and SILT in the healthy colon**

It has been shown for the formation of secondary lymphoid tissue, during embryonic life, that a lymphotoxin independent clustering of lymphoid tissue inducer (LTi) cells takes place before LTβR triggering starts, which gives rise to a rudimentary lymph node anlagen. At embryonic day 14.5 (E14.5)
both wild type and LTα⁻/- animals show comparable clustering of LTi cells, forming rudimentary lymph node anlagen. At E16.5 in wild type animals the peripheral lymph node anlagen continue to form whereas in LTα⁻/-LTi cells fail to continue clustering and the lymph node anlagen dissociates. These data indicate the presence of a lymphotoxin independent pathway for the initiation of secondary lymphoid tissue formation. The extent to which this applies to the formation of secondary lymphoid tissue and SILT in the setting of colon remained to be investigated. In chapter 2 we show that indeed clusters of LTi cells are present in the colon before birth. These structures occur in constant number along the antimesenteric border in the submucosa of the colon. Over the ensuing two weeks after birth these anlagen mature and fill with T and B cells contained in separate micro domains and referred to as colonic patches which appear to be the counterpart of PPs occurring in the small intestine. The formation of both PPs and MLNs have been shown to be dependent on the LTα₁β₂-LTβR signaling pathway as these structures cannot be found in the intestine of adult LTα⁻/- and LTβR⁻/- mice. The reported absence of colonic patches in the adult colon of LTα⁻/- mice implies a vital role for the LTα₁β₂-LTβR signaling axis in the formation of these structures. In chapter 2 we confirm the findings that colonic patches are indeed absent in the adult colon of LTα⁻/- mice. Interestingly, we clearly show that clusters of LTi cells are present before birth and disappear after birth, indicating a lymphotoxin independent mechanism for the initial clustering of LTi cells in the colon, similar to what is observed for lymph node formation. We also found that the more immature stages of SILT (commonly referred to as cryptopatches) form in the colon two weeks after birth and are still present in the adult colon. These inducible lymphoid organs have also been shown to be dependent on the LTα₁β₂-LTβR signaling as more mature SILT cannot be detected in the small intestine of LTα⁻/- mice. We confirmed for the colon that indeed LTα⁻/- adult mice lack more mature classes of SILT containing B cells (also referred to as mature ILFs) yet they do form immature SILT containing LTi like Rorγt⁺ CD4⁺ CD3⁻ cells. This observation further supports the hypothesis that there is a lymphotoxin independent pathway which is responsible for the initial formation of organized GALT. It is also tempting to speculate that this lymphotoxin independent pathway precedes the LTα₁β₂-LTβR signaling axis
and is involved in the initiation of SILT formation.

**The lymphotoxin independent pathway – Neurons provide a source of retinoic acid in the formation of secondary lymphoid tissue**

In chapter 3 we showed that a lymphotoxin independent pathway exit in the context of the formation of peripheral lymph nodes where neurons are a potential a source of retinoic acid (RA). This RA is required for the induction of CXCL13 in stromal cells which serves to attract LTi cells to the area. This initiating event gives rise to the first clustering of LTi cells in a lymphotoxin independent manner. Subsequently, \( \text{LT}\alpha_{1}\beta_{2} - \text{LT}\beta R \) triggering is needed to maintain these structures and form secondary lymphoid tissue. This provides a remarkable insight into the formation of secondary lymphoid tissue as this could mean that RA derived form a neuronal source can lead to the induction of chemokines needed for the clustering of hematopoetic cells (Figure 1). It is important to bear in mind that many cells in the intestine have the ability to provide a source of RA as they contain vitamin A conversion enzymes, retinaldehyde dehydrogenase (RALDH). It has been shown that intestinal epithelial cells, lamina propria mesenchymal cells, dendritic cells and macrophages also contain RALDH enzymes and they may also serve as a source of RA \(^{10-12}\). However in the context of the adult intestine we indeed show that stimulation of the vagal nerve, which innervates the intestine, can cause an induction of CXCL13 expression which can be blocked by RAR\(\beta \) specific inhibitors. This implicates vitamin A and its active metabolite retinoic acid (RA), coming from a neuronal source, as a potential alternate pathway for the formation of organized GALT.

**Tertiary lymphoid tissue in chronically inflamed colon - a lymphotoxin independent signaling pathway**

Tertiary lymphoid tissue develops in adult life in a variety of different chronic inflammatory diseases and it is thought that the formation of tertiary lymphoid tissue, like secondary lymphoid tissue, is also dependent on the \( \text{LT}\alpha - \text{LT}\beta R \) signaling pathway \(^{7,13,14}\). It has been shown that inducible brochus associated tissue (iBALT), tertiary lymphoid tissue which forms due to inflammation in the lungs, can form independently of the \( \text{LT}\alpha_{1}\beta_{2} - \text{LT}\beta R \) signaling pathway \(^{15}\).
Figure 1 – Formation of secondary lymphoid tissue - the lymphotoxin independent pathway

Neuronal fibres occur in close proximity to stromal organizer cells. These neuronal fibres provide a source of retinoic acid (RA) which leads to an increased expression of the chemokine CXCL13 by these stromal organizer cells. This increased expression of CXCL13 attracts the first lymphoid tissue inducer (LTi cells) to the area. The subsequent triggering of LTβR on stromal organiser cells by Ltαβ2 expressed by LTi cells will then cause up regulation of additional chemokines (CCL21 and CCL19) and adhesion molecules (ICAM-1, VCAM-1 and MAdCAM-1). These chemokines and adhesion molecules serve to attract and retain more LTi cells to the area, which in turn leads to more receptor triggering, subsequently giving rise to a self sustaining feedback loop responsible for the formation of a lymph node.

Due to the fact that two types of organized GALT i.e. colonic patches and SILT already exit in the healthy colon a clear definition of these structures was necessary to be able to detect the formation of additional tertiary lymphoid tissue in the setting of the inflamed colon. In chapter 2 we showed that colonic patch anlagen were present before birth as clusters of LTi cells and that these structures remained constant in number and are colonized by B and T cells in the first two weeks after birth. These colonic patches in the adult setting occur in the sub mucosa of the colon and contain distinct T and B cell areas. SILT on the other hand are present within the lamina propria and consist of a single B cell follicle containing scattered T cells. In chapter 4 we showed that indeed a third type of organized GALT emerges, which can only be found in the context of the chronic inflammatory setting. These structures can clearly be distinguished from secondary lymphoid tissue and SILT present in the healthy colon due to the fact that they form in the lamina propria and consist of more than one B cell follicle with distinct T cell areas. Intriguingly,
this tertiary lymphoid tissue is also found in LTα−/− which suffer from chronic inflammation of the colon, and thus confirm a similar lymphotoxin independent pathway for the formation of tertiary lymphoid tissue in the context of the inflamed colon. Furthermore, in chapter 3 we show that neurons may provide a source of RA which can cause stromal cells to upregulate CXCL13, which can attract B cells to these tertiary lymphoid structures. Since modulation of the vagal nerve resulted in an RA dependent upregulation of CXCL13 in the intestine of adult mice it is an intriguing idea that perhaps, in analogy with embryonic development, RA derived from nerve fibres could in principle initiate the formation of tertiary lymphoid tissue in the inflamed colon. To support this hypothesis preliminary stainings were performed on tertiary lymphoid tissue of wild type and LTα−/− mice for neuronal markers and RALDH. Indeed, these initial stainings show that neurons, which express RALDH, are present within the tertiary lymphoid tissue of both wild type and LTα−/− mice. This illustrates that indeed neurons through their ability to convert vitamin A to RA could play a role in the formation of tertiary lymphoid tissue in the context of the inflamed intestine. To test this hypothesis, future experiments are needed to determine whether indeed neurons contribute to the formation of tertiary lymphoid tissue in inflamed colon. To eliminate the neuronal source of RA selective denervation experiments should be performed, after which the formation of tertiary lymphoid tissue can be induced through administration of DSS. These experiments are beyond the scope of this study but will be performed in the future.

The role of vitamin A and retinoic acid in the formation of organized GALT

In chapter 5 we show that the level of RA mediated signaling is higher in the small intestines of BALB/c mice when compared to C57BL/6 mice. By quantifying the expression of RARβ, which is reflective of the amount of RA mediated signaling since RARβ is a direct target gene of RA, we showed that more RA mediated signaling occurs in the small intestine of BALB/c compared to C57BL/6 mice. Indeed, BALB/c mice showed enhanced levels of RARβ mRNA in the intestines compared to C57BL/6 mice. Furthermore, we showed that BALB/c mice display more organized GALT in the small intestine
compared to C57BL/6. We hypothesised that this increase in organized GALT tissue would be of benefit in the context of clearing inflammatory responses in the context of the intestine since regulatory T cells and IgA producing B cells could be detected here. To ultimately show whether the levels of RA mediated signaling could affect the formation of organized GALT one would have to address this in a suitable mouse model by inducing this pathway experimentally by oral administration of RA and quantifying organized GALT.

The role of vitamin A and retinoic acid in intestinal inflammation
Ulcerative colitis and Crohn's Disease, collectively referred to as inflammatory bowel disease (IBD), are chronic inflammatory diseases which affect the intestines. Dextran sulfate sodium (DSS) induced colitis is a commonly used animal model, which gives rise to intestinal inflammation reflecting ulcerative colitis. Numerous research groups have shown that BALB/c mice suffer from a less severe form of DSS induced colitis than C57BL/6 mice, however the underlying reason for this is not known. Due to the fact that DSS induced colitis primarily affects the colon we investigated in chapter 6 the differences in organized GALT of the colon, RALDH and RARβ expression levels of BALB/c and C57BL/6 mice. We show that BALB/c mice also have an increased amount of organized GALT along with increased expression levels of RALDH1 and RARβ in the colon. DSS colitis was induced in these two mouse strains and we confirmed that BALB/c mice suffer from a less severe colitis even when higher percentages of DSS are given to this mouse strain. To confirm that vitamin A and its active metabolite RA were crucial for controlling the mucosal immune response during colitis, we induced colitis in vitamin A deficient animals. Animals on a vitamin A deficient diet showed an increase in the severity of DSS induced colitis compared to controls, further supporting that vitamin A and its metabolic break down product RA are important for regulating the mucosal immune system during colitis. Vitamin A deficiency is commonly associated with IBD patients due to the fact that they suffer from malabsorption due to chronic inflammation of the intestinal tract. Patients with IBD have been shown to suffer from increased intestinal permeability which would enhance chronic inflammation due to a
decreased epithelial barrier integrity \textsuperscript{21,22}. Interestingly, it has been shown \textit{in vitro} that RA can upregulate the expression of tight junction molecules and thus have a beneficial effect in IBD by restoring epithelial barrier integrity \textsuperscript{23}. In animals models for inflammatory bowel disease vitamin A deficiency has been shown to increase the severity of disease \textsuperscript{24}. On the other hand, RA supplementation has been shown to ameliorate disease in animal models for IBD \textsuperscript{25}. Colonic biopsies taken from patients and cultured with RA showed a decrease in IL-17 and an increase in FoxP3 expression, indicating that the administration of RA to patients with IBD may have an anti-inflammatory effect of these patients \textsuperscript{26}. It may thus be important to verify whether intake of vitamin A, and / or conversion to RA, occurs at normal levels in IBD patients. If perhaps this is affected, either by malabsorption or by genetic defect leading to the decreased metabolism of vitamin A, then supplementation of vitamin A or RA may be a potential therapeutic strategy for treating IBD. Vitamin A supplementation in patients suffering from a variety of other chronic inflammatory disorders i.e. skin disorders such as acne vulgaris, broncho-pulmonary dysplasia and some forms of cancer has already been shown to have a beneficial anti-inflammatory effect in these patients \textsuperscript{27}. RA supplementation is also currently being used as a treatment for some cancer patients \textsuperscript{28}. Taken together this indicates that vitamin A, or its active metabolite RA, shows exciting potential as a therapy in IBD.

\textbf{Tertiary lymphoid tissue - beneficial or harmful?}

The consequences of the formation of tertiary lymphoid tissue in inflammation and its involvement in immunity is largely unknown. The formation of tertiary lymphoid tissue, in which self antigen is constitutively presented and autoreactive T cells are stimulated, will maintain and further contribute to auto immune reactions \textsuperscript{29,30}. Interestingly, in the context of the mucosal environment there is evidence to support that tertiary lymphoid tissue may actually be of benefit to the host in responding to disease. It has been shown that mice with iBALT structures clear influenza virus at a more rapid rate than mice lacking these structures, indicating a protective role for lung associated tertiary lymphoid tissue \textsuperscript{15,31,32}. It has been shown that iBALT structures contain germinal centres (GCs) which serve as an additional
ectopic site for the production of IgA \(^3\). In general terms one can state that iBALT structures serve the purpose of bringing organized lymphoid tissue closer to the invading pathogen. In chapter 6 we show that IgA producing B cells along with regulatory T cells, which serve to suppress inflammation, are largely contained in organized GALT. Preliminary stainings confirm that the B cell areas of tertiary lymphoid tissue contain IgA producing B cells and that the T cell areas of tertiary lymphoid tissue also contain T regulatory cells (data not shown). Thus, tertiary lymphoid tissue may be of benefit to the host by providing an additional ectopic environment which contains IgA producing B cells regulatory T cells and.

**Future perspective - The role of vitamin A and its active retinoic acid as a treatment in IBD patients**

Inflammatory bowel disease is a debilitating disease which effects approximately 2.2 million people in Europe \(^3\). The social impact on the individual as well as the economic impact on the individual and society is vast \(^3\). Thus the need for better therapies to treat the disease is necessary. As discussed previously administration of vitamin A or its active metabolite RA may serve as a potential new therapy for IBD patients. If indeed tertiary lymphoid tissue is of benefit in intestinal inflammation another potential therapy would be to try and enhance the formation of this lymphoid tissue in patients with IBD. We have shown that modulation of the vagal nerve can cause a RA dependent increase of CXCL13 in the setting of the adult mouse intestine. This increase may provide a local source of RA, and thus CXCL13, in the lamina propria and thereby enhance the formation of tertiary lymphoid tissue in chronic inflammation. If indeed tertiary lymphoid tissue is of benefit in dealing with chronic inflammation in the intestine, by providing an additional ectopic site for IgA production and regulatory T cells, it would be of great benefit if one could promote the formation of these structures in IBD patients. Further investigation of this topic is required however it does give rise to the intriguing concept that vagal nerve stimulations of patients may help to alleviate disease. Evidence in animal models supports this idea as it has been shown that by cutting the vagal nerve supply to the intestine a more severe form of colitis develops in these animals \(^3\). Vagal nerve stimulations
are currently being used as a treatment in patients suffering from epilepsy and depression\textsuperscript{39,40}. The idea that vagal nerve stimulations could be used as a possible treatment for patients suffering from inflammation of the intestinal tract has been proposed as a viable new potential therapy in treating these patients \textsuperscript{41,42}. Thus these data taken together provide a novel approach of vagal nerve stimulations as a possible treatment with patients suffering from IBD.
Reference List


